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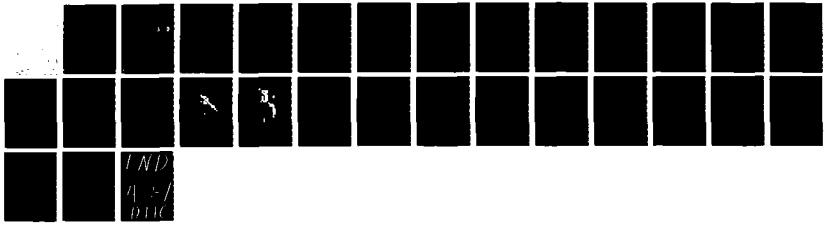

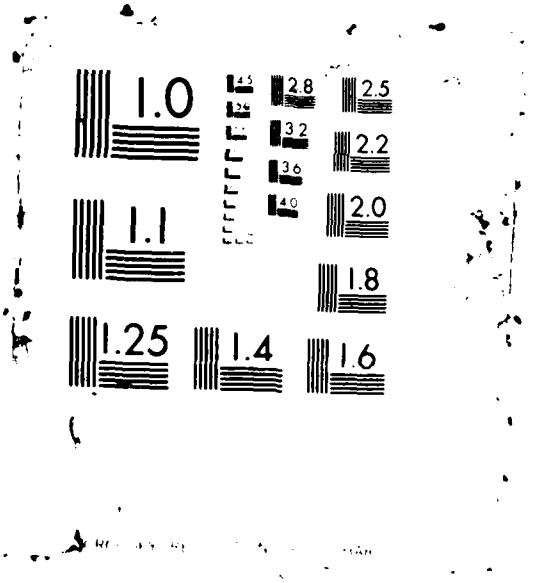
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Mechanisms of Bunyavirus Virulence: A Genetic Approach

Annual/ Final Report

Neal Nathanson, M.D.

September 1986

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20. (Abstract)

The construction and characterization of hybridomas making monoclonal antibodies against La Crosse and Tahyna viruses is described. These antibodies have been characterized by the following tests: (1) ELISA; (2) neutralization; (3) hemagglutination inhibition (HI); (4) cross reactivity against California serogroup viruses; (5) immunoprecipitation of viral proteins; (6) isotyping; and (7) fusion inhibition (FI). These antibodies have been shown to be useful tools for the classification of new viral isolates and for the phenotyping of reassortant viruses. They have been used to select variant viruses which can escape neutralization. An epitope map has been constructed which shows that there are a series of overlapping epitope clusters within a single antigenic site. A preliminary survey has been conducted of the protective ability of these antibodies in mice.

The virulence of California serogroup viruses is being studied by genetic analysis. (a) Two parent viruses have been selected to represent a virulent prototype (La Crosse original) and an avirulent prototype (Tahyna 181/57). (b) The pathogenesis of these two viruses has been studied by infection of suckling mice, and it has been found that the neuroinvasive virulent virus replicates well in striated muscle with subsequent viremia, while the avirulent virus does not. (c) Reassortants have been constructed from these two parents and have been genotyped. Virulence is under polygenic control, such that the M RNA segment is the major determinant while the other two segments modulate virulence on certain genetic backgrounds. (d) Variant viruses have been tested for their neuroinvasiveness in mice. Variants at one epitope site exhibit reduced mouse virulence.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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1. Introductory note: Scope and Duration

This contract operated from 11-1-81 through 11-30-85, or four years 1 month. This report was written in July 1986, after termination of the contract and includes is all of the work conducted under the contract. Investigators collaborating on this program are listed below:

Neal Nathanson	Professor	Pathogenesis and immunology
Francisco-Gonzalez	Assistant Professor	Hybridomas and variant viruses

(a) Papers

Much of our work is summarized in 10 papers (8 published, 2 in press) which are attached to this report as Appendices 1-10. The body of the report focuses on the highlights of the data. Details and techniques are available in the appendices.

(b) Overview

The focus of the contract was the development, characterization, and utilization of hybridomas and monoclonal antibodies. This work was a component of a larger program devoted to molecular determinants of the virulence of La Crosse bunyaviruses.

2. Hybridomas against La Crosse and Tahyna Viruses

(a) Uses of hybridomas

The central theme of our studies is to use the California virus system as a model to study the factors which determine the outcome of acute viral encephalitis. Among these factors are virus determinants (neuroinvasiveness and neurovirulence) and host determinants (recovery from infection and protection against subsequent infection). We have used monoclonal antibodies as a tool to investigate several aspects of this model.

(i) To identify and map biological functions of the viral glycoproteins. Important functions associated with glycoproteins of enveloped viruses are: binding to erythrocytes (hemagglutination), binding to host cells (neutralization), and fusion of membranes (hemolysis or cell fusion).

(ii) The monoclonal antibodies may be grouped into epitope clusters, in one of several ways. The epitope clusters then can be used to construct an antigenic map of individual proteins, such as the external glycoproteins of La Crosse virus.

(iii) To select antigenic variants of parental virus. Growth in the presence of monoclones will select efficiently for variant viruses. If variants show altered virulence, this provides evidence that the altered viral protein is an important determinant of virulence. Variants with altered virulence can be studied for other biological functions, such as fusion, which may correlate with in vivo properties.

(iv) To determine the protective role of antibodies directed against antigenic determinants of California encephalitis virus glycoproteins.

In other enveloped viruses antibodies against certain sites on the glycoprotein will neutralize, but the efficiency may differ markedly, depending on whether the site is involved in attachment or in fusion. Also, there may be a synergistic effect of neutralizing antibodies against two different antigenic sites.

Antibodies which fail to neutralize may have several different effects: in some cases non-neutralizing antibodies block neutralization, while in other instances they are protective in vivo.

Finally, the properties of the antibody itself (avidity, complement fixation, ability to mediate virolysis and cytolysis, and the like) may influence its protective efficiency. Such questions can be studied with monoclonal antibodies much more precisely than could ever be accomplished with polyclonal antisera.

(b) Immunization of mice and construction of hybridomas

La Crosse and Tahyna viruses were selected for this study because they represent antigenically distinct strains with relative differences in virulence, LaCrosse being the more neuroinvasive (kills after ip injection) and Tahyna the more avirulent (fails to kill after ip injection above 2 weeks of age).

To immunize mice, advantage was taken of the fact that La Crosse and Tahyna viruses produce active infections in mice. Several weeks later a booster injection of virus was given; 2-4 days later mice were sacrificed and spleen cells prepared.

Spleen cells from LAC or TAH immunized mice were fused with a BALB/c myeloma line (P3 x 63 clone 653) which is a nonsecretor. A mixture of spleen:myeloma cells at a 10:1 ratio was made and PEG 1000 used as fusing agent. The mixture was plated in micro wells, 5×10^5 cells per well.

After 2-3 weeks of incubation, wells with visible colonies were tested for anti-viral antibody in ELISA assay, using partially purified virus as antigen. Positive cultures were cloned in 0.25% agarose and individual colonies were transferred to flasks and again tested for antibody. Hybridoma cells were maintained in 15% serum and supernates collected as a cell culture source of monoclonal antibody. For high titer preparations, 10^7 hybridoma cells were injected ip into Pristane-primed BALB/c mice and ascitic fluids collected 1-2 weeks later. Antibody titers of ascitic fluids were usually about 100-fold higher than titers of tissue cultures supernates. For neutralization, HI, CF, and ELISA, the ascitic fluid served well, but tissue culture supernate or purified immunoglobulin was required for clean immunoprecipitations. Cells were stored in a serum-DMSO mixture in liquid N2.

(c) Characterization of monoclonal antibodies

To characterize the LAC and TAH hybridomas, each monoclone was used to immunoprecipitate virus proteins from an S35 amino acid labelled lysate of infected cells, and each was typed as to immunoglobulin class by RIA. In addition, each antibody was tested in ELISA, neutralization (N), and hemagglutination (HI) systems, against each of 11 California serogroup viruses. The essential results are set forth in TABLES 1 and 2. TABLE 1 may be summarized:

(i) Of 23 Monoclones, 15 were directed against the G1 glycoprotein and 8 against the N nucleoprotein, while none were against the G2 glycoprotein or the L polypeptide.

(ii) Of the 15 G1 clones, 11 both neutralized and had HI activity, one had HI activity only, and 3 were neutralization and HI negative. From this it was inferred that the G1 glycoprotein had at least two antigenic sites. One site is postulated to bind to receptors on both erythrocytes and BHK cells, accounting for the concordance of neutralization and HI results. The other site appeared uninvolved in attachment to receptors. More recent epitope studies (see below) suggest that there is only one antigenic site for both groups of antibodies.

(iii) Of the 15 G1 clones 4 were type-specific i.e., reacted with the immunizing virus only, 3 were almost type-specific, while 8 were cross-reactive.

(iv) None of the 8 nucleocapsid clones showed neutralizing or HI activity, as expected.

(v) Of the 8 nucleocapsid clones, only one was type-specific, while 7 were cross-reactive.

(vi) The 15 G1 clones were isotyped as IgG1 (7 clones), IgG2a (6 clones), or IgG2b (2 clones). By contrast, the 8 nucleocapsid clones were IgM (5 clones), Ig2a (2 clones), or undetermined (1 clone).

(d) Variant viruses selected with neutralizing monoclonal antibodies specific for the G1 glycoprotein

One established application of monoclonal antibodies is the selection of variant viruses. Such variants may then be used for: (i) grouping of epitopes into natigenic sites; (ii) for the mapping of sequential epitopes onto the amino acid sequence of the G1 glycoprotein; and (iii) the use of variants to delineate submolecular determinants of virus virulence.

F. Gonzalez has selected a series of 35 variants using 11 neutralizing monoclonal antibodies, according to standard methods. These variants are summarized in TABLE3. In addition, he has selected some "double" variants, by sequential passage of parent virus in the presence of two different antibodies.

The single variants have been used for studies of virulence. Of the 11 variants tested (TABLE 5), three have shown attenuation in mice. One of these, V22, has been studied in some detail, as described below. Single variants have also been used for the mapping of epitopes, as described in the following section.

(e) Classification of epitopes on the G1 glycoprotein with monoclonal antibodies and variant viruses

The glycoproteins of enveloped viruses have several important biological functions including attachment to cellular receptors, fusion neutralization, and (in some instances) neuraminidase activity. It has been long established that the California serogroup of bunyaviruses agglutinate erythrocytes and are readily neutralized. Recently, we have shown that these viruses can also mediate fusion from without or within.

As an approach to study of these glycoprotein functions, we isolated a panel of hybridomas and selected anti-G1 monoclonal antibodies. A subset of these antibodies mediatd neutralization and hemagglutination-inhibition; since there was almost complete concordance between the two activities, it appeared that the same domain on the G1 protein was involved in attachment to BHK cells and erythrocytes. In addition, these antibodies appeared to define a second domain on the G1 protein which was not involved in either neutralization or HI activity.

In view of the importance of the biological activities of the G1 protein, it was of interest to determine whether the neutralizing monoclonal antibodies defined one or several antigenic sites. We elected to approach this question by testing a panel of variant viruses agains the monoclonal antibodies used to select them. A total of 35 variants, representing 10 different antibodies, were plaque-purified. These were then tested in two systems: (1) cross-neutralization of the variants against the panel of selecting monoclones; and (2) an radiommunoassay (RIA) to compare the ability of antigens made from the parent La Crosse and the 10 variants to bind both neutralizing and non-neutralizing anti-G1 monoclonal antibodies.

TABLE I

Characterization of 23 monoclonal antibodies against La Crosse (LAC) and Tahyna (TAH) viruses

Clone No.	Immunizing Virus	Protein Precipitated	Ig Class	Type-Specific or Cross-Reactive	Serologic Test			
					ELISA	NT	HI	FI
807-09	LAC	GI	IgG2a	S	+	+	+	+/-
807-15	LAC	GI	IgG2b	S	+	+	+	+
807-18	LAC	GI	IgG1	S	+	+	+	+
807-35	LAC	GI	IgG1	S	+	+	+	+
807-31	LAC	GI	IgG1	S	+	+	+	+
807-12	LAC	GI	IgG2a	C	+	+	+	+
807-22	LAC	GI	IgG2a	C	+	+	+	+
807-33	LAC	GI	IgG2a	C	+	+	+	+
807-25	LAC	GI	IgG2b	S	+	+	?	+
807-26	LAC	GI	IgG2a	S	+	-	-	-
807-21	LAC	GI	IgG2a	C	+	-	-	-
807-13	TAH	GI	IgG1	C	+	+	+	?
813-48	TAH	GI	IgG1	C	+	+	+	-
813-77	TAH	GI	IgG1	C	+	+	+	ND
814-443	TAH	GI	IgG1	C	+	-	+	-
820-374	LAC	N	IgM	C	+	-	+	-
807-28	LAC	N	IgG2a	C	+	-	-	ND
807-32	LAC	N	IgM	C	+	-	-	ND
307-13	LAC	N	IgM	C	+	-	-	-
807-02	TAH	N	IgG2a	S	+	-	-	ND
814-08	TAH	N	?	C	+	-	-	ND
814-48	TAH	N	IgM	C	+	-	-	ND
814-87	TAH	N	IgM	C	+	-	-	ND

N: nucleocapsid. NT: neutralization test. HI: hemagglutination inhibition test. FI: fusion inhibition. S: type-specific. C: cross-reactive. Fusion inhibition: a 1:50 dilution of ascitic fluid gives a fusion index of 0.70 or lower, compared to a control index of 0.90 or higher. Antibody 807-25 was originally classified as non-neutralizing but later ascitic fluids were clearly neutralizing. Antibody 807-13 was originally neutralizing but later ascitic fluids show minimal neutralizing activity.

TABLE 2

Additional 21 monoclonal antibodies against the G1 protein of LAC and TAH viruses (data of F. Gonzalez-Scarano, J. Gentsch, and N. Pobjecky, 1985)

Clone No.	Immunu-nizing Virus	Protein Precipi-tated	Serological Test ELISA	NT	HI	FI
807-05	LAC	G1	+	+	+	-
807-07	LAC	G1	+	-	*	ND
807-17	LAC	G1	+	-	*	-
813-57	TAH	G1	+	-	*	-
813-71	TAH	G1	+	-	*	-
813-72	TAH	G1	+	-	*	ND
814-91	TAH	G1	+	-	*	-
820-260	LAC	G1	+	+	*	+
900-03	LAC	G1	+	+	*	ND
900-27	LAC	G1	+	+	*	+/-
900-62	LAC	G1	+	+	*	+/-
900-04	LAC	G1	+	-	*	ND
900-05	LAC	G1	+	-	*	ND
900-08	LAC	G1	+	-	*	ND
900-11	LAC	G1	+	-	*	ND
900-13	LAC	G1	+	-	*	ND
900-19	LAC	G1	+	-	*	ND
900-25	LAC	G1	+	-	*	-
900-29	LAC	G1	+	-	*	-
900-61	LAC	G1	+	-	*	-
900-69	LAC	G1	+	-	*	-

* HI: not yet run. Isotype and cross-reactivity to be determined. NT: neutralization test. FI: fusion inhibition.

TABLE 3

Neutralizing anti-G1 protein monoclonal antibodies and the frequency and virulence of antigenic variants selected by them*

Epitope	Antibody	Immunizing Virus	Specific (S) or Cross-Reactive (C)	Frequency of Variants log $_{10}$)	Virulence
1	807-31	LAC	S	-6.0	+
	807-09	LAC	S	-3.7	+
	807-35	LAC	S	-5.3	+
	807-13	TAH	C	-5.9	+
	807-18	LAC	S	-5.3	+
	807-15	LAC	S	-6.2	+
2	829-60	LAC	ND	-5.4	+
3	807-12	LAC	C	-5.0	R
	807-33	LAC	C	-4.1	R
4	807-25	LAC	ND	-6.4	+
5	807-22	LAC	C	-6.3	R

* Virulence was based on subcutaneous injection of suckling mice with 1000 pfu (+: as virulent as parent virus; R: reduced by comparison with parent virus).

Neutralization assays. Cross-neutralization tests were done with each of the 11 monoclonal antibodies against 1-6 variants per eptitope. Representative results are summarized in Fig. 1. The results were scored as neutralization, partial neutralization, or no neutralization, as defined in the caption. With the exception of antibodies 807-12 and 807-33, all of the monoclonals showed distinct patterns of cross-neutralization. The neutralization antibodies fall into five distinct groupings (TABLE 3), with three of the groupings represented by a single antibody. The largest group, headed by antibody 807-31, is mainly composed of antibodies that are strain specific, and it represents the major neutralizing region on G1. Antibodies 807-12 and 807-33 appear identical by this analysis, but in fact showed differences when a panel of California viruses was used in neutralization assays with them.

ELISA. The 11 variant viruses (one variant per epitope) were tested against the 11 neutralizing monoclonal antibodies plus 8 non-neutralizing monoclonal antibodies against the G1 protein. Binding to parent LAC virus was defined as 100% and binding to variants was scored relative to this standard, after equalizing the amount of each variant used. Binding to variants ranged from 0% to 100% (or occasionally more than 100%), providing dramatic differences and the clear cut patterns in Fig. 2.

Almost all of the monoclonals showed significantly reduced binding by ELISA to the variant viruses selected with them; for most of the antibodies there was, in fact, no binding above background (Fig. 2). Antibodies 807-9 and 807-22 bound the variants selected with them when tested in ELISA, in spite of their inability to neutralize these variants. For antibody 807-22 this binding was only 69% of the binding parent LAC, but antibody 807-9 bound the variant selected with it as well as it bound parent LAC.

Many of the monoclonal antibodies also demonstrated reduced or no binding to variants selected with other antibodies, producing a pattern of interrelationships. The results obtained with ELISA are in excellent agreement with the findings of the neutralization tests (Fig. 2), although there are some differences with the map obtained in the cross-neutralization assays. There are five groups of antibodies that include at least one neutralizing monoclonal. The largest cluster, again headed by antibody 807-31, now consists of 9 monoclonals, since three non-neutralizing antibodies map to this area. The group headed by antibody 807-22 now also includes three non-neutralizing antibodies that show diminished binding to the variant (22) obtained with it. These four antibodies also demonstrate reduced binding to acid-treated virus. We had previously proposed that they comprise a cluster of epitopes which is affected by the conformational change that G1 undergoes at the pH of fusion.

Variant 25, which had appeared isolated in cross-neutralization assays, now appears closely related to variant 22, since it shows reduced binding of the four antibodies defining epitope 2. In addition, epitope 25 now is related to two other groups of antibodies (those headed by 807-31 and 807-12).

Comparison of neutralization and ELISA results. A comparison of the binding and cross-neutralization data is presented in TABLE 4. Over 85% (23/27) of the antibody-virus combinations that showed no neutralization also showed decreased binding in ELISA (0-74%). Similarily, 95% (88/92) of the antibody-virus combinations that neutralized showed binding on ELISA that was close to control values (75-100%).

Turning to apparent inconsistencies, there were four instances where neutralizing antibodies failed to neutralize variants to which they bound strongly (75%-100%), i.e., antibody 807-09 (variant 9), antibody 807-18 (variants 31 and 35), and antibody 807-15 (variant 13). A similar phenomenon is illustrated by the 8 non-neutralizing antibodies, most of which apparently bind to neutralizing epitopes. Similar observations have been reported for other viruses, and there may be several explanations, such as low antibody-antigen affinity or multiple steps in the antibody-virus interaction leading to neutralization.

There are two instances where antibodies that neutralized a variant did not bind it on ELISA. That apparent inconsistency occured only with variant 18 (and antibodies 807-9 and 807-15). It seems likely that the procedure used to coat virus onto ELISA plates, including alkaline pH, altered the conformation of epitope 18.

Summary. This analysis of the antigenic structure of the G1 glycoprotein of LAC virus leads to two major conclusions. (i) The epitopes identified by monoclonal antibodies can be separated into distinct groups or clusters. The neutralization assays define 5 such clusters, and the ELISA data suggest that there may be a sixth cluster defined by non-neutralizing antibodies 807-21 and 807-26. (ii) The data suggest that these 6 epitopes clusters may be a part of a single immunodominant antigenic site, since the ELISA results indicate interrelationships between all of the groups, with the exception of the single monoclonal antibody 820-260. Of particular importance, the data do not suggest that there is a separate non-neutralizing antigenic site as previously postulated.

(f) Demonstration that La Crosse virus mediates pH-dependent fusion

Glycoproteins of enveloped viruses mediate several important biological functions. These are: (i) attachment to cellular receptors; (ii) attachment to erythrocytes; (iii) fusion of viral envelope to cellular membranes; and (iv) neuraminidase or other enzymatic activity. Of the functions listed above, the putative fusion function of bunyavirus glycoproteins had not been demonstrated prior to our studies.

Over the past few years, many enveloped viruses have been shown to have lipid binding and membrane fusing properties which become manifest only upon exposure of the virions to acidic pH. These properties may be necessary in order for virions to extrude their nucleocapsids into the cellular cytoplasm following uptake by endocytosis into acidic lysosomal vacuoles.

Although fusion of virion envelopes with cellular membranes can be visualized with the electron microscope, routine demonstration of fusion by this method is difficult. Therefore, a variety of indirect measures of fusion have been introduced to characterize the parameters of this glycoprotein function. Among these, low pH mediated hemolysis of red blood cells, and cell-to-cell fusion of tissue culture cells are the simplest. Cell-to-cell fusion may employ virus absorbed on cells (fusion from without or FFWO) or viral glycoproteins expressed on the surface of infected cells (fusion from within or FFWI).

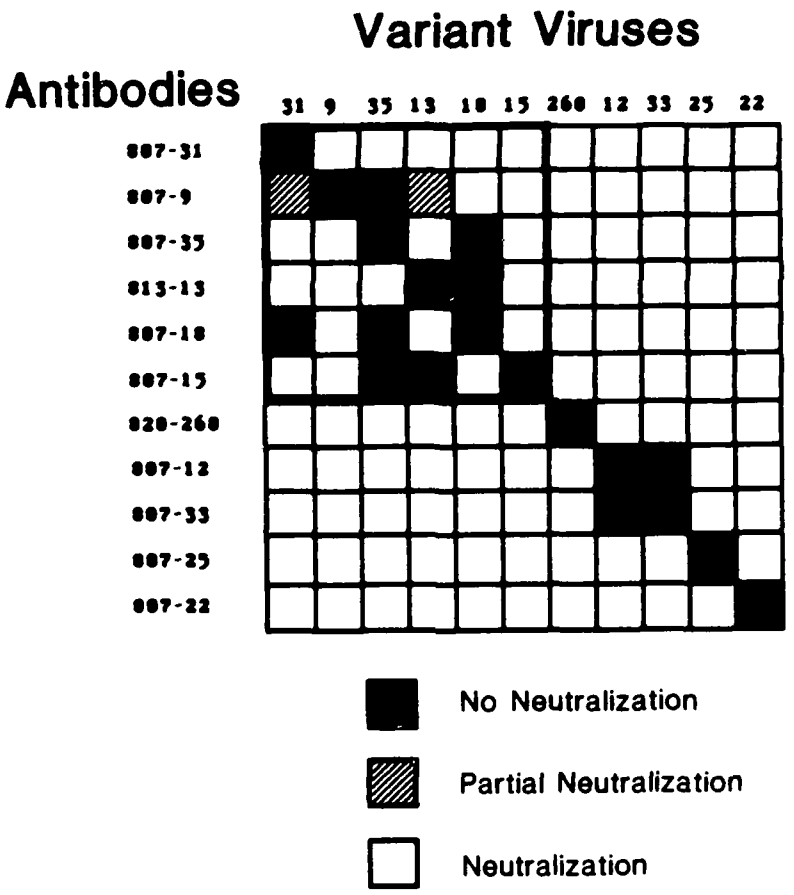

Fig. 1. Neutralization of antigenic variants by the monoclonal antibodies used to select them. The 11 antibodies shown neutralized parent La Crosse virus at titers ranging from 1:320 to 1:100,000. No neutralization was defined as an neutralization titer less than 1:20. Neutralization was defined as a titer against a variant virus which was no less than 2-fold below the titer of the same antibody against present La Crosse virus. Partial neutralization was seen only for antibody 807-09.

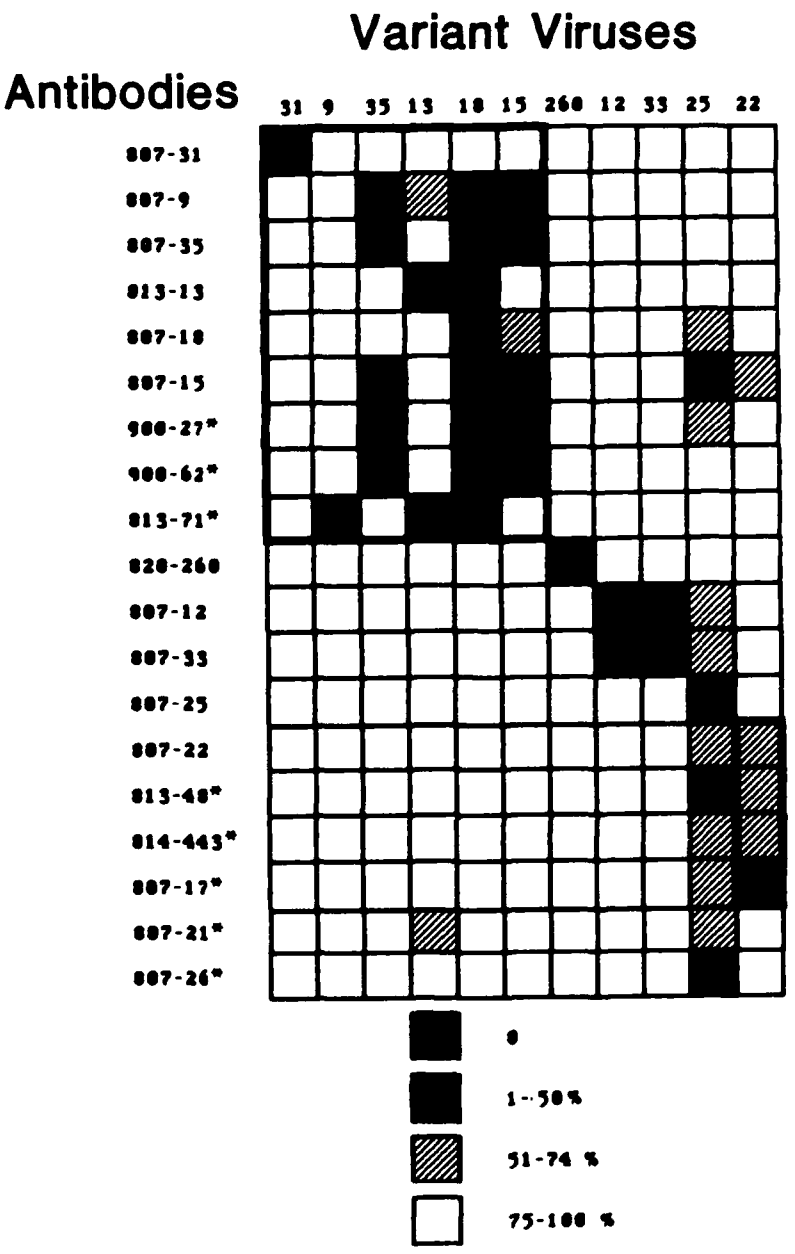

Fig. 2. Binding of anti-G1 glycoprotein monoclonal antibodies to antigenic variants in ELISA. Binding to variant viruses is recorded relative to binding of the same antibody to parent La Crosse virus. Values greater than 100% were grouped in the 75-100% category. Binding in the 1-50% category was almost always in the 20-50% range, with two exceptions. Asterisk (*) indicates non-neutralizing antibodies.

TABLE 4

A comparison of neutralization and binding (ELISA) tests on 11 neutralizing monoclonal antibodies and the 11 variant viruses selected by them*

Neutralization	ELISA 0%	ELISA 1-74%	ELISA 75-100%	Totals
None	14	9	4	27
Partial	0	1	1	2
Complete	2	2	88	92
Totals	16	12	93	121

* Based on data in Figures 1 and 2.

Cell fusion (FFWO) was demonstrated by adaptation of the method of White and colleagues. Gradient-purified La Crosse virus was added to uninfected BHK cells and maintained at 4C for one hour, following which the inoculum was removed. The cells were briefly (30-60 seconds) exposed to pre-warmed MEM with 0.2% BSA containing 10 mM Hepes and 10 mM Morpholinopropanesulfonic acid (MOPS) or Morpholinoethanesulfonic acid (MES) buffer adjusted to the appropriate pH with NaOH. The buffer was removed and the cells incubated at 37C under neutral pH for 30-60 minutes, after which the monolayer was fixed and stained.

The fusion index equals (1-N/C) where N is the number of nuclei and C is the number of cells. The index was determined by directly counting representative fields. At 37C and 30 minutes, maximum fusion was obtained at pH 5.0-6.0. The amount of virus used in each experiment was a critical determinant of the extent of fusion. The temperature at which the cells were incubated following exposure to low pH was important also. At low temperatures, cell fusion does not proceed at all, and it is slow at 31.5C. Fusion also occurs with virus that has been inactivated by exposure to ultraviolet light to reduce the PFU titer by 100,000-fold.

Fusion from within (FFWI). Fusion has now been demonstrated to occur as a consequence of infection (FFWI) in our laboratory. BHK-21 cells are infected with an moi of 1.0, and incubated 16 hours at 35C. The infected monolayer is washed with PBS and incubated with MOPS buffer, for 30 minutes, at 37C. At pH between 5.0 and 6.0 dramatic fusion occurs (index of 0.8 or greater).

In collaboration with Dr. Jonathan Smith, USAMRIID, we have looked at FFWI in the electron microscope. The images provide a vivid demonstration of the "melting" away of plasma membranes during the fusion process. At high magnification virions can be seen between apposed plasma membranes, and it is unclear whether these extracellular virions are responsible for "FFWI". Alternatively, G1 and G2 synthesized within infected cells and transported to the plasma membrane, could be capable of mediating FFWI.

By analogy with other systems, the demonstration that LAC has a pH dependent fusion function extends the putative entry pathway involving acidic vesicles to another family of enveloped viruses.

Blocking of fusion with monoclonal antibodies. The FFWI system has made it possible to determine whether anti-G1 monoclonal antibodies are capable of blocking the fusion function, after virus has attached to the cell surface. BHK-21 monolayers, 16 hours after infection, are washed and overlaid with dilutions of antibody. After 30 minutes incubation at pH7, MOPS buffer is added at pH 5.5 and 37C, and the index determined 30 minutes later. Antibodies are capable of blocking fusion, and there appears to be a general correlation between anti-fusion activity and neutralization titer

(g) Conformational alteration in the G1 glycoprotein at reduced pH

The activation of fusion function in the orthomyxoviridae is associated with a conformational change of the hemagglutinin molecule which occurs upon exposure of the virions to an acid environment. A similar phenomenon can be demonstrated with La

Crosse G1 glycoprotein. Tryptic cleavage of purified virions at 23C results in cleavage of G1 into a major peptide of about 95kD (and no cleavage of either N or G2), and fragments of low molecular weight that are not resolved in a 12.5% acrylamide gel. If prior to protease treatment the virions are exposed to an acidic environment, the peptide pattern is different, and the G1 is now cleaved into a 82Kd fragment as well as peptides in the 10-15Kd range. The pH range that results in different cleavage pattens correlates with the pH range that leads to activation of the fusion function.

Alteration of the cleavage pattern following exposure of the virus to an acid environment, can be demonstrated with a variety of proteases, including chymotrypsin, fibrinolysin, elastase, bromelain, and pronase. The minor glycoprotein, G2, is not as susceptible to protease cleavage as G1. Following exposure to acid (pH 5.8), G2 is partially cleaved by fibroinolysin. In a series of timed reactions, this cleavage is seen to occur only after G1 has been extensively degraded, suggesting that the major glycoprotein may protect G2.

Additional evidence of the presence of a conformational change in the G1 glycoprotein at the pH of activation of the fusion function comes from studies with monoclonal antibodies. As part of the characterization of epitopes, monoclonal antibodies against the G1 protein were tested in an ELISA assay with virus that had been treated with acid. Antibody 807-17 demonstrates reduced binding to virus that has been acid-treated. Antibody 807-18, also illustrated, shows the more typical pattern, that is no difference in the binding of virus that has been exposed to acid. Control ascites fluids consisted of antibodies to the nucleocapsid protein.

G1 (120 kilodaltons) and G2 (34 kilodaltons) are present in approximately equimolar amounts in the virion envelope. Either molecule, or both, could be involved in FFWO. G1 undergoes a conformational change at acidic pH altering both tryptic cleavage and antigenic sites but, in the absence of sequence information, it is not possible to predict whether it, or G2, contains the fusion peptide.

(h) Protection of mice with monoclonal antibodies

One goal of our studies is to utilize monoclonal antibodies to analyze the epitopes involved in protective immunity against La Crosse and related bunyaviruses.

We explored several alternative protocols for in vivo virus challenge to be used to screen for antibody mediated protection. It was considered desirable to (i) challenge by a peripheral route rather than by ic injection, in order to mimic a realistic challenge and to insure ability to detect protection; (ii) to use a modest virus dose to enhance ability to detect protection; (iii) to produce 100% mortality so that the screen could be carried out with a small number of mice; (iv) to administer antibody before challenge and by a different route to avoid an "in vivo neutralization test" and to mimic pre-exposure immunization. We concluded that the use of sc infection of suckling mice with 100 PFU (4LD100) would best meet the foregoing criteria, with antibody given ip 1 day earlier.

TABLE 5

Correlation of anti-fusion activity of monoclonal antibodies and neutralization titer for anti-G1 protein antibodies*

Antibody Number	Neutralization Activity	HI Activity	Epitope Group	Log_{10} Neutralization Titer*	Fusion Inhibition Activity
807-31	+	+	1	> 4.6	3+
807-25	+	+	4	> 4.6	3+
807-33	+	+	3	4.0	2+
807-260	+	ND	2	4.0	2+
807-12	+	+	3	4.0	2+
807-35	+	+	1	3.7	3+
807-18	+	+	1	3.4	2+
807-09	+	+	1	2.8	+/-
807-22	+	+	5	2.8	2+
807-15	+	+	1	2.8	1+
900-27	+	ND	ND	2.5	+/-
900-62	+	ND	ND	2.5	+/-
813-48	+	+	4/5	2.2	+/-
813-71*	+?	ND	1	1.6	-
807-05	-	ND	ND	< 1.3	-
807-13*	-?	-	ND	< 1.3	-
807-17	-	ND	4/5	< 1.3	-
807-21	-	-	4	< 1.3	-
807-26	-	-	4	< 1.3	-
813-57	-	-	ND	< 1.3	-
814-443	-	ND	4/5	< 1.3	-
814-91	-	+	ND	< 1.3	-
900-25	-	ND	ND	< 1.3	-
900-29	-	ND	ND	< 1.3	-
900-61	-	ND	ND	< 1.3	-
900-69	-	ND	ND	< 1.3	-

* Neutralization titer and fusion inhibition activity on same ascitic fluids. FI activity: - index 0.9; +/-, index 0.9; +, index 0.7-0.9; ++, index 0.4-0.7; +++ index 0.4. Antibodies needing a check: 813-71 borderline neutralization; 807-13 previously a neutralizing antibody. FI: data of N. Pobjecky, 1985.

We elected to screen a large number of antibodies first, rather than test a few antibodies in detail. The results of this screen are presented in TABLE 6. Several tentative conclusions can be drawn: (i) the test protocol works well and yields 100% mortality (0% protection) in controls, while protective antibodies give 0% mortality (100% protection). (ii) when tested in a single concentration of 1:8, clear cut results emerge which suggest that antibodies with neutralizing activity protect while those which are neutralization test negative fail to protect in vivo. (iii) there is no evidence that HI or FI activity is independently relevant to protection since antibodies which are N-HI+ (814-443) fail to protect and antibodies which are N+ FI- (813-71) do protect. (iv) non-neutralizing antibodies which react with neutralizing epitopes 4 or 5 fail to protect (ie, 807-21 or 807-26). (v) individual monoclonal antibodies against single epitopes seem quite competent to mediate in vivo protection.

(i) Further questions

The data reported above represent solid progress in the characterization of La Crosse and Tahyna monoclonal antibodies. The results suggest a number of questions which could be answered in future studies. Some of these questions are briefly outlined below.

(i) Continuation of studies on grouping and mapping the G1 monoclonal antibodies. With the completion of epitope analysis of variant viruses, the focus will shift to physical mapping of the epitopes of the G1 protein. This is bring attacked by cloning and sequencing the segment of the M RNA which encodes G1. This is a preliminary to sequencing variant viruses to localize those epitopes which represent sequential antigenic determinants.

(ii) Construction of G2 hybridomas.

(iii) The use of monoclonal antibodies to determine the role of G1 and G2 proteins in fusion. Results to date indicate that G1 plays a critical role in fusion; the importance of G2 remains to be determined.

(iv) Determination of the potential protective role of neutralizing and non-neutralizing anti-G1 antibodies. Protection can now be correlated with neutralization, HI, and anti-fusion activities. Preliminary results indicate that all neutralizing monoclonal antibodies are potentially protective; such antibodies are fusion inhibiting FI also. It would now be possible to look in detail at selected monoclonal antibodies (perhaps column purified) to ask (a) whether protection is quantitatively related to plaque reduction titer or to FI titer; (b) whether protection is unrelated to HI activity; (c) whether protection relates to isotype; and (d) whether non-neutralizing antibodies can block protection conferred by neutralizing antibodies which bind to the same epitope.

(v) Ultimately, if some protective monoclonal antibodies can be mapped to specific tryptic peptides, it would be potentially possible to sequence such peptides, synthesize them, and test them as immunogens.

TABLE 6

Protection of suckling mice given an ip dose of 0.05 ml of 91:8 concentration of monoclonal antibody and challenged 1 day later with 800 pfu (100 LD50) of La Crosse virus by the sc route (0.05 ml)

Protein Precipitated	Epitope Group	Antibody Number	Serotesting NT*	HI	FI*	Percent Protection
GI	1	807-15	2.8	+	+	100%
	1	807-18	3.4	+	++	100%
	1	807-31	4.0	+	+++	100%
	1	807-35	4.3	+	+++	83%
	2	820-260	4.0	ND	++	100%
	3	807-12	4.3	+	++	100%
	3	807-33	3.1	+	++	100%
	4	807-25	4.3	+	+++	100%
	5	807-22	2.5	+	++	17%
	4/5	813-48	3.7	+	+/-	67%
	1	813-71	2.5	ND	-	100%
	ND	813-77	2.5	+	ND	100%
	1	900-27	2.8	ND	+/-	100%
	ND	807-01	1.0	ND	ND	0%
	ND	807-07	1.0	ND	ND	0%
	4/5	807-17	1.0	ND	-	0%
	4	807-21	1.0	-	-	0%
	4	807-26	1.0	-	-	0%
	ND	813-72	1.0	ND	ND	0%
	4/5	814-443	1.0	+	-	0%
	ND	900-11	1.0	ND	ND	0%
N		807-32	1.0	-	ND	0%
		814-02	1.0	-	ND	0%
		814-87	1.0	-	ND	0%
		820-260	1.0	-	ND	0%

* For epitope grouping see TABLE 4. NT: neutralization tests run on same ascites tap tested for protection. HI: hemagglutination inhibiton tests on a different ascites tap. FI: fusion inhibition test, where a single dilution of 1:50 was tested and the fusion index recorded as less than 0.40 (+++), 0.40-0.70 (++), 0.70-0.90 (+), 0.90 (+/-) or greater than 0.9 (-). Protection: based on mortality 2 weeks after challenge. 807-13: ascites fluid used for protection had no neutralizing activity although earlier taps were NT positive.

3. Genes and proteins which determine the virulence of California serogroup viruses

The primary thrust of this project was the characterization and utilization of hybridomas. However, it was part of a larger program which is devoted to a definition of the molecular determinants of the virulence of California serogroup viruses. The next few pages provides a brief summary of our studies of virulence determinants, some of which utilized monoclonal antibodies.

(a) Polygenic Control of the virulence of reassortants between La Crosse and Tahyna viruses

La Crosse and Tahyna viruses were selected as prototypes of virulent and avirulent strains, respectively. Tahyna/181-57 virus exhibits markedly reduced neuroinvasiveness, even in suckling mice, in comparison with La Crosse/original virus. The PFU/LD50 ratio of Tahyna virus is about 30,000-fold higher than the ratio for La Crosse virus.

Reassortants were constructed by co-infecting BHK cell cultures with both viruses and plaquing the progeny. To quickly screen individual clones, each one was labelled with S35 methionine and the proteins electrophoresed on polyacrylamide gels; minor but consistent differences in the migration of G1 and N proteins were exploited to rapidly phenctype the M RNA segment, encoding the G1 protein, and the S RNA segment, encoding the N protein.

Selected clones were then genotyped by RNA-RNA hybridization. Reference viruses, ie, the parental La Crosse and Tahyna strains, were labelled with P32, the RNA extracted, and hybridized with RNA from cell lysates prepared from individual virus clones. After hybridization, the RNA was digested with S1 nuclease, which cuts single stranded nucleic acids. Hybrids between labelled and unlabelled La Crosse or labelled and unlabelled Tahyna RANs are protected, while heterologous hybrids are digested by the enzyme. The products were electrophoresed on agarose gels, and the individual full length strands were identified in autoradiographs. The method, although cumbersome, gave clearcut genotypes.

A panel of reassortants representing all possible genotypes was assembled, with 2-3 clones per genotype. All clones were assayed for their PFU/LD50 ratios, and classified as virulent, avirulent, or intermediate in virulence. The results showed that when the M RNA segment was derived from Tahyna virus, the reassortants were about 10-fold more virulent than the avirulent parent Tahyna virus. Pathogenesis studies of a selected reassortant confirmed the intermediate virulence phenotype. Thus, an avirulent genetic background, the L and S RNA segments modulated avirulence.

(b) Avirulent of selected monoclonal antibody resistant (MAR) variant viruses

Another approach to characterization of the role of viral gene segments in virulence is to examine the effect of point mutations in specific proteins upon virulence. We have used variant viruses selected by specific monoclonal antibodies to

obtain mutants in the G1 glycoprotein. F. Gonzalez selected a numbe of such mutants with a panel of different neutralizing antibodies. The epitopes identified by these antibodies were then classified into clusters, as described above.

When the variant viruses were tested for virulence, variants at two of 5 epitope groups were found to show reduced virulence, and this was particularly true for variant 22, at epitope 807-22. This variant showed reduced peripheral virulence as well as modest reduction in intracerebral virulence. Of interest, there was a correlation between variants with reduced virulence and variants which exhibited an altered fusion profile.

In summary, these data indicated that the G1 protein played a role in determination of virulence, consistent with the genetic studies which indicated that the M RNA segment, encoding the G1 protein, was a major determinant of virulence. Furthermore, the results suggested that the fusion function which is critical for the early steps in cellular infection, might play a role in the determination of virulence in vivo.

(c) Future questions

The present results have indicated a number of directions for future studies of the molecular basis of virulence of California serogroup viruses. These include:

Variant viruses

(i) Selection of antigenic revertants, particularly from the V22 variant, to determine whether such revertants show a reversion in biological properties, such as virulence and fusion.

(ii) Selection of variants by serial passage through several different monoclonal antibodies, to determine the biological impact of accumulated point mutations in the G1 protein.

(iii) Selection of variants of Tahyna/181-57 viurs to determine whether they show an increase or decrease in virulence.

Biological markers

(iv) Additional biological markers for characterizing viruses should be identified, such as infectivity for mosquitoes by feeding or by intrathoracic injection. (Collaborative studies with Barry Beaty indicate that this is feasible.)

(v) Characterization of additional prototype viruses with biological marker profiles different from parent La Crosse and Tahyna viruses. Candidates are the V22 variant, and the PP31 clone of La Crosse virus. These viruses may show reduced infectivity for mosquitoes compared to La Crosse and Tahyna viruses. In addition, it would be useful to select a virus with reduced intracerebral virulence for mice. Biological marker profiles will provide evidence whether there are multiple genetic loci which determine virus virulence.

(vi) Construction of reassortants between a few selected viruses with different biological marker profiles to determine the gene segments associated with each marker. If all markers map to the M RNA segment, it would be useful to select recombinant viruses within the M RNA segment.

Improved methods

(vii) An improved method for rapid genotyping, such as blot hybridization with synthetic oligonucleotides, would be very useful.

(vii) A myotube culture system, in which the myoblasts are differentiated into myotubes, should be explored to see if it reflects the in vivo differences between La Crosse/original and Tahyna/181-57 viruses.

Molecular mapping of biologically relevant sites on the G1 molecule

(xi) Cloning the M RNA segment of La Crosse virus.

(x) Determination of the RNA sequence of the M RNA segment and the amino acid sequence of the NH_4 terminus of the G1 protein, making it possible to deduce the amino acid sequence of the G1 protein.

(xi) Mapping the G1 mutation sites of a few selected variants, emphasizing those with biologically interesting changes. This could be done by sequencing the relevant portions of the M RNA segment of each variant virus.

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